Development and Characterization of Recombinant Adenoviruses Encoding Human p53 for Gene Therapy of Cancer

KEN N. WILLS, DANIEL C. MANEVAL, PATRICIA MENZEL, MATTHEW P. HARRIS,
SUGANTO SUTJIPTO, MEI-TING VAILLANCOURT, WHEI-MEI HUANG,
DUANE E. JOHNSON, SCOTT C. ANDERSON, SHU FEN WEN, ROBERT BOOKSTEIN,
H. MICHAEL SHEPARD, and RICHARD J. GREGORY

ABSTRACT

We have constructed recombinant human adenoviruses that express wild-type human p53 under the control of either the Ad 2 major late promoter (MLP) or the human cytomegalovirus (CMV) immediate early gene promoter. Each construct replaces the Ad 5 E1a and E1b coding sequences necessary for viral replication with the p53 cDNA and MLP or CMV promoter. These p53/Ad recombinants are able to express p53 protein in a dose-dependent manner in infected human cancer cells. Tumor suppressor activity of the expressed p53 protein was assayed by several methods. [^{3}H]Thymidine incorporation assays showed that the recombinant adenoviruses were capable of inhibiting DNA synthesis in a p53-specific, dose-dependent fashion. Ex vivo treatment of Saos-2 tumor cells, followed by injection of the treated cells into nude mice, led to complete tumor suppression using the MLP/p53 recombinant. Following a single injection of CMV/p53 recombinant adenovirus into the peritumoral space surrounding an in vivo established tumor derived from a human small cell lung carcinoma cell line (NIH-H69), we were able to detect p53 mRNA in the tumors at 2 and 7 days post-injection. Continued treatment of established H69 tumors with MLP/p53 recombinant led to reduced tumor growth and increased survival time compared to control treated animals. These results indicate that recombinant adenoviruses expressing wild-type p53 may be useful vectors for gene therapy of human cancer.

OVERVIEW SUMMARY

Introduction of the p53 tumor suppressor gene into tumors bearing p53 mutations can inhibit cellular proliferation and tumorigenicity. Wills et al. describe replication-deficient recombinant adenoviruses directing expression of human p53 both in vitro and in vivo. They show that adenovirus-mediated expression of wild-type p53 in p53 altered tumors can suppress proliferation and inhibit tumorigenicity in ex vivo and in vivo cancer models.

INTRODUCTION

MUTATION OF THE P53 GENE is the most common genetic alteration in human cancers (Bartek et al., 1991; Holl-

stein et al., 1991). In its proposed role as a "guardian of the genome" (Lane, 1992), the p53 gene product functions as a transcriptional activator of other genes which inhibit cell cycle progression from G_1 to S phase in normal cells. Its levels rise and accumulate in response to DNA damage, leading either to G_1 arrest and repair, terminal differentiation, or, if too much damage has occurred, apoptosis (Kuerbitz et al., 1992; Lane, 1992). Loss of wild-type p53 function is associated with the uncontrolled growth of many types of human cancers. The reexpression of normal p53 in p53-altered tumor cells has been demonstrated to suppress tumor growth (Chen et al., 1990; Cheng et al. 1992; Takahashi et al., 1992) or induce apoptosis (Yonish-Rouach et al., 1991, Shaw et al., 1992). Therefore, p53 functions as a tumor suppressor, restoring a nontumorigenic phenotype to tumor cells in which the endogenous p53 gene has been deleted or mutated.

CANJI, Inc., San Diego, CA 92121

Recent work has shown that human adenoviruses can be used to deliver genes successfully into a variety of cells and tissues (Lemarchand et al., 1992; Rosenfeld et al., 1992; Rich et al., 1993). Recombinant adenoviruses have several advantages over alternative gene delivery systems such as retrovirus (RV) or adeno-associated virus (AAV)-based vectors for the treatment of cancer. These include the ability to produce stable, high-titer virus capable of efficient infection and subsequent gene expression in target cells (for review, see Siegfried, 1993). Because of the advantages of an adenovirus-based delivery system over other systems for the potential gene therapy of cancer, we constructed recombinant adenoviruses encoding wild-type p53 under the control of the Ad 2 major late promoter (MLP) or the human cytomegalovirus (CMV) promoter. We have tested the ability of these constructs to suppress tumor growth both in vitro and in vivo.

MATERIALS AND METHODS

Cell lines

Recombinant adenoviruses were grown and propagated in the human embryonal kidney cell line 293 (ATCC CRL 1573) maintained in DME medium containing 10% defined, supplemented calf serum (Hyclone). Saos-2 cells were maintained in Kaighn's media supplemented with 15% fetal calf serum. HeLa and Hep 3B cells were maintained in DME medium supplemented with 10% fetal calf serum. All other cell lines were grown in Kaighn's media supplemented with 10% fetal calf serum. Saos-2 cells were kindly provided by Dr. Eric Stanbridge. All other cell lines were obtained from ATCC.

Construction of recombinant adenoviruses

To construct the Ad5/p53 viruses, a 1.4-kb Hind III–Sma I fragment containing the full-length cDNA for p53 was isolated from pGEM1-p53-B-T (kindly supplied by Dr. Wen-Hwa Lee) and inserted into the multiple cloning site of the expression vector pSP72 (Promega) using standard cloning procedures (Sambrook et al., 1989). The p53 insert was recovered from this vector following digestion with Xho I–Bgl II and gel electrophoresis. The p53 coding sequence was then inserted into either pNL3C or pNL3CMV adenovirus gene transfer vectors (kindly provided by Dr. Robert Schneider), which contain the Ad5 5' inverted terminal repeat and viral packaging signals and the E1a enhancer upstream of either the Ad2 major late promoter (MLP) or the human cytomegalovirus immediate early gene promoter (CMV), followed by the tripartite leader cDNA and Ad 5 sequence 3,325–5,525 bp in a pML2 background. These new constructs replace the E1 region (bp 360–3,325) of Ad5 with p53 driven by either the Ad2 MLP (A/M/53) or the human CMV promoter (A/C/53), both followed by the tripartite leader cDNA (see Fig. 1). The p53 inserts use the remaining downstream E1b polyadenylation site. Additional MLP- and CMV-driven p53 recombinants (A/M/N/53, A/C/N/53) were generated which had a further 705-nucleotide deletion of Ad 5 sequence to remove the protein IX (pIX) coding region. As a control, a recombinant adenovirus was generated from the parental pNL3C plasmid without a p53 insert (A/M). A second
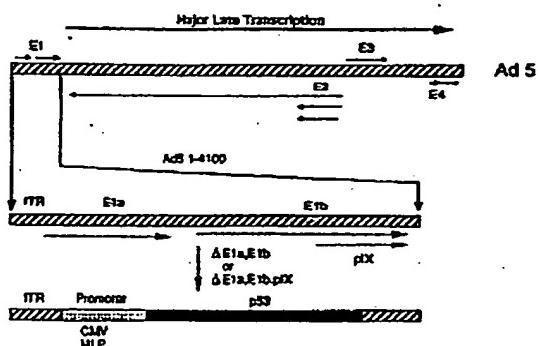

FIG. 1. Schematic of recombinant p53/adenovirus constructs. The p53 recombinants are based on Ad 5 and have had the E1 region of nucleotides 360–3,325 replaced with a 1.4-kb full-length p53 cDNA driven by the Ad 2 MLP (A/M/53) or human CMV (A/C/53) promoters followed by the Ad 2 tripartite leader cDNA. The control virus A/M has the same Ad 5 deletions as the A/M/53 virus, but lacks the 1.4-kb p53 cDNA insert. The remaining E1b sequence (705 nucleotides) have been deleted to create the protein IX-deleted constructs A/M/N/53 and A/C/N/53. These constructs also have a 1.9-kb Xba I deletion within adenovirus type 5 region E3.

control (kindly provided by Dr. Robert Schneider) consisted of a recombinant adenovirus encoding the β-galactosidase (β-Gal) gene under the control of the CMV promoter (A/C/β-Gal). The plasmids were linearized with either Nru I or Eco RI and cotransfected with the large fragment of a Cla I-digested Ad 5 dl309 or dl327 mutants (Jones and Shenk, 1979; Thimmappaya et al., 1982) using a Ca/PO₄ transfection kit (Stratagene). Only the pIX-minus constructs used the dl327 background which contains a 1.9-kb Xba I deletion in the E3 region. Viral plaques were isolated and recombinants identified by both restriction digest analysis and the polymerase chain reaction (PCR) using recombinant-specific primers against the tripartite leader cDNA sequence with downstream p53 cDNA sequence. Recombinant virus was further purified by limiting dilution, and virus particles were purified and titered by standard methods (Graham and van der Erb, 1973; Graham and Prevec, 1991).

p53 protein detection

Saos-2 or Hep 3B cells (5 × 10⁵) were infected with the indicated recombinant adenoviruses for a period of 24 hr at increasing multiplicities of infection (moi) of plaque-forming units of virus/cell. Purified adenovirus, stored in 1% sucrose in phosphate-buffered saline (PBS), is diluted with media to obtain the desired moi and added to plates of cells containing fresh media. After 24 hr, the cells were washed once with PBS and harvested in lysis buffer [50 mM Tris-HCl pH 7.5, 250 mM NaCl, 0.1% NP-40, 50 mM NaF, 5 mM EDTA, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride (PMSF)]. A Bradford assay (Bio-Rad Protein Assay kit) was used to measure cellular protein concentration, and equal amounts of protein (approximately 30 μg) were separated

p53 RECOMBINANT ADENOVIRUS GENE THERAPY OF CANCER   1081

by 10% SDS-PAGE and transfered to nitrocellulose. Membranes were incubated with α-p53 antibody PAb 1801 (Novocastro) followed by sheep anti-mouse IgG conjugated with horseradish peroxidase. p53 protein was visualized by chemiluminescence (ECL kit, Amersham) on Kodak XAR-5 film.

Measurement of DNA synthesis rate

Cells (5×10^3/well) were plated in 96-well titer plates (Costar) and allowed to attach overnight ($37°C$, 7% CO_2). Cells were then infected for 24 hr with purified recombinant virus particles at moi values ranging from 0.3 to 100, as indicated. Media were changed 24 hr after infection, and incubation was continued for a total of 72 hr. [^3H]Thymidine (Amersham, 1 μCi/well) was added 18 hr prior to harvest. Cells were harvested on glass fiber filters and levels of incorporated radioactivity were measured in a beta scintillation counter. [^3H]Thymidine incorporation was expressed as the mean % ($\pm$SD) of media control and plotted versus the moi.

Tumorigenicity in nude mice

Approximately 2.4×10^8 Saos-2 cells, plated in T225 flasks, were treated with suspension buffer (1% sucrose in PBS) containing either A/M/N/53- or A/M-purified virus at an moi of 3 or 30. Following an overnight infection, cells were injected subcutaneously into the left and right flanks of BALB/c athymic nude mice (4 mice per group). One flank was injected with the A/M/N/53-treated cells, while the contralateral flank was injected with the control A/M-treated cells, each mouse serving as its own control. Animals receiving bilateral injection of buffer-treated cells served as additional controls. Tumor dimensions (length, width, and height) and body weights were then measured twice per week over an 8-week period. Tumor volumes were estimated for each animal, assuming a spherical geometry with radius equal to one-half the average of the measured tumor dimensions.

Intratumoral RNA analysis

Female BALB/c athymic nude mice (approximately 5 weeks of age) were injected subcutaneously with 1×10^7 H69 small cell lung carcinoma (SCLC) cells in a 200-μl volume in their right flanks. Tumors were then allowed to progress for 32 days. Mice then received peritumoral injections of either A/C/53 or A/C/β-Gal recombinant adenovirus [2×10^9 plaque-forming units (pfu)] into the subcutaneous space beneath the tumor mass. Tumors were excised from the animals 2 and 7 days post adenovirus treatment and rinsed with PBS. Tumor samples were homogenized, and total RNA was isolated using a TriReagent kit (Molecular Research Center, Inc.). Poly(A) RNA was isolated using the PolyATract mRNA Isolation System (Promega), and approximately 10 ng of sample was used for reverse transcriptase (RT)-PCR determination of recombinant p53 mRNA expression (Wang et al., 1989). Primers were designed to amplify sequence between the adenovirus tripartite leader cDNA and the downstream p53 cDNA, ensuring that only recombinant, and not endogenous p53 would be amplified.

p53 gene therapy of established tumors in nude mice

Approximately 1×10^7 H69 (SCLC) tumor cells in 200-μl volumes were injected subcutaneously into female BALB/c athymic nude mice. Tumors were allowed to develop for 2 weeks, at which point animals were randomized by tumor size ($n = 5$/group). Peritumoral injections of either A/M/N/53 or the control A/M adenovirus (2×10^9 pfu/injection) or buffer alone (1% sucrose in PBS) were administered twice per week for a total of 8 doses/animal per group. Tumor dimensions and body weights were measured twice per week for 7 weeks, and tumor volume was estimated as described previously. Animals were then followed to observe the effect of treatment on mouse survival.

RESULTS

Construction of recombinant p53-adenovirus

p53 adenoviruses were constructed by replacing a portion of the E1a and E1b region of adenovirus type 5 with p53 cDNA under the control of either the Ad2 MLP (A/M/53) or CMV (A/C/53) promoter (schematized in Fig. 1). This E1 substitution severely impairs the ability of the recombinant adenoviruses to replicate, restricting their propagation to 293 cells that supply Ad 5 E1 gene products in trans (Graham et al., 1977). After identification of p53 recombinant adenovirus by both restriction digest and PCR analysis, the entire p53 cDNA sequence from one of the recombinant adenoviruses (A/M/53) was sequenced to verify that it was free of mutations. Following this, purified preparations of the p53 recombinants were used to infect HeLa cells to assay for the presence of phenotypically wild-type adenovirus. HeLa cells, which are nonpermissive for replication of E1-deleted adenovirus, were infected with 1–4 $\times 10^9$ infectious units of recombinant adenovirus at an moi = 50, cultured for 3 weeks, and observed for the appearance of cytopathic effect (CPE). Using this assay, we were not able to detect recombinant adenovirus replication or wild-type contamination, readily evident by the CPE observed in control cells infected with wild-type adenovirus at a level of sensitivity of approximately 1 in 10^9.

p53 protein expression from recombinant adenovirus

To determine if our p53 recombinant adenoviruses expressed p53 protein, we infected tumor cell lines that do not express endogenous p53 protein. The human tumor cell lines Saos-2 (osteosarcoma) and Hep 3B (hepatocellular carcinoma), which contain mutations that result in no expression of p53 protein (Chen et al., 1990; Hsu et al., 1993), were infected for 24 hr with the p53 recombinant adenoviruses A/M/53 or A/C/53 at moi values ranging from 0.1 to 200 pfu/cell. Western analysis of lysates prepared from infected cells demonstrated a dose-dependent p53 protein expression in both cell types (Fig. 2). Both cell lines expressed higher levels of p53 protein following infection with A/C/53 than with A/M/53 (Fig. 2). No p53 protein was detected in noninfected cells. Cells infected with moi values of up to 200 of the control virus A/M also did not show detectable p53 protein (unpublished observation). SW 480 cell

A.
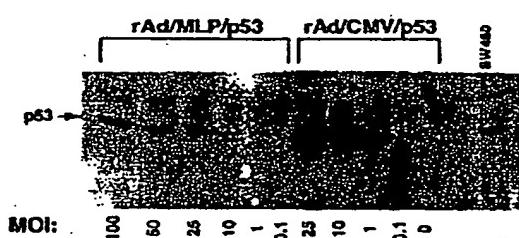
B.
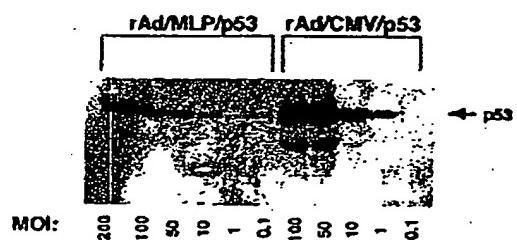
FIG. 2. p53 protein expression in tumor cells infected with A/M/53 and A/C/53. A. Saos-2 (osteosarcoma) cells were infected at the indicated moi with either the A/M/53- or A/C/53-purified virus and harvested 24 hr later. The p53 antibody pAb 1801 was used to stain immunoblots of samples loaded at equal total protein concentrations. Equal protein concentrations of SW480 cell extracts, which overexpress mutant p53 protein, were used as a marker for p53 size. The zero (0) under the A/C/53 heading indicates a mock infection containing untreated Saos-2 lysate. B. Hep 3B (hepatocellular carcinoma) cells were infected with the A/M/53 or A/C/53 virus at the indicated moi and analyzed as in A. The arrow indicates the position of the p53 protein.

lysate, which overexpresses mutant p53 protein (Baker et al., 1990), was used as a size marker. Levels of endogenous wild-type p53 are normally quite low, and nearly undetectable by Western analysis of cell extracts (Bartek et al., 1991). It is clear however that wild-type p53 protein levels are easily detectable after infection with either A/M/53 or A/C/53 at the lower moi values (Fig. 2), suggesting that even low doses of p53 recombinant adenoviruses can produce potentially efficacious levels of p53.

p53-dependent morphology changes

The reintroduction of wild-type p53 into the p53-negative osteosarcoma cell line, Saos-2, results in a characteristic enlargement and flattening of these normally spindle-shaped cells (Chen et al., 1990). Subconfluent Saos-2 cells (1 × 10⁵ cells/10-cm plate) were infected at an moi of 50 with either the A/C/53 or control A/M virus, and incubated at 37°C for 72 hr until uninfected control plates were confluent. At this point, the expected morphological change was evident in the A/C/53-treated plate (Fig. 3C), but not in uninfected (Fig. 3A) or control virus-infected plates (Fig. 3B). This effect was not a function of cell density because a control plate initially seeded at lower density retained normal morphology at 72 hr when its

confluence approximated that of the A/C/53-treated plate (data not shown). Our previous results had demonstrated a high level of p53 protein expression at a moi of 50 in Saos-2 cells (Fig. 2A), and these results provided evidence that the p53 protein expressed by these recombinant adenoviruses was biologically active.

p53 inhibition of cellular DNA synthesis

To test further the activity of the p53 recombinant adenoviruses, we assayed their ability to inhibit proliferation of human tumor cells as measured by the uptake of [³H]thymidine. It has previously been shown that introduction of wild-type p53 into cells that do not express endogenous wild-type p53 can arrest the cells at the G₁/S transition, leading to inhibition of uptake of labeled thymidine into newly synthesized DNA (Baker et al., 1990; Diller et al., 1990; Mercer et al., 1990). We infected a variety of p53-deficient tumor cell lines with either A/M/N/53, A/C/N/53 or a non-p53-expressing control recombinant adenovirus (A/M). We observed a strong, dose-dependent inhibition of DNA synthesis by both the A/M/N/53 and A/C/N/53 recombinants in 7 out of the 9 different tumor cell lines tested (Fig. 4). Both constructs were able to inhibit DNA synthesis specifically in these human tumor cells, regardless of whether they ex-

FIG. 3. p53-dependent Saos-2 morphology change. Subconfluent (1 × 10⁵ cells/10-cm plate) Saos-2 cells were either uninfected (A), infected at a moi = 50 with the control A/M virus (B), or the A/C/53 virus (C). The cells were photographed 72 hr post-infection.

Multiplicity of Infection (MOI)
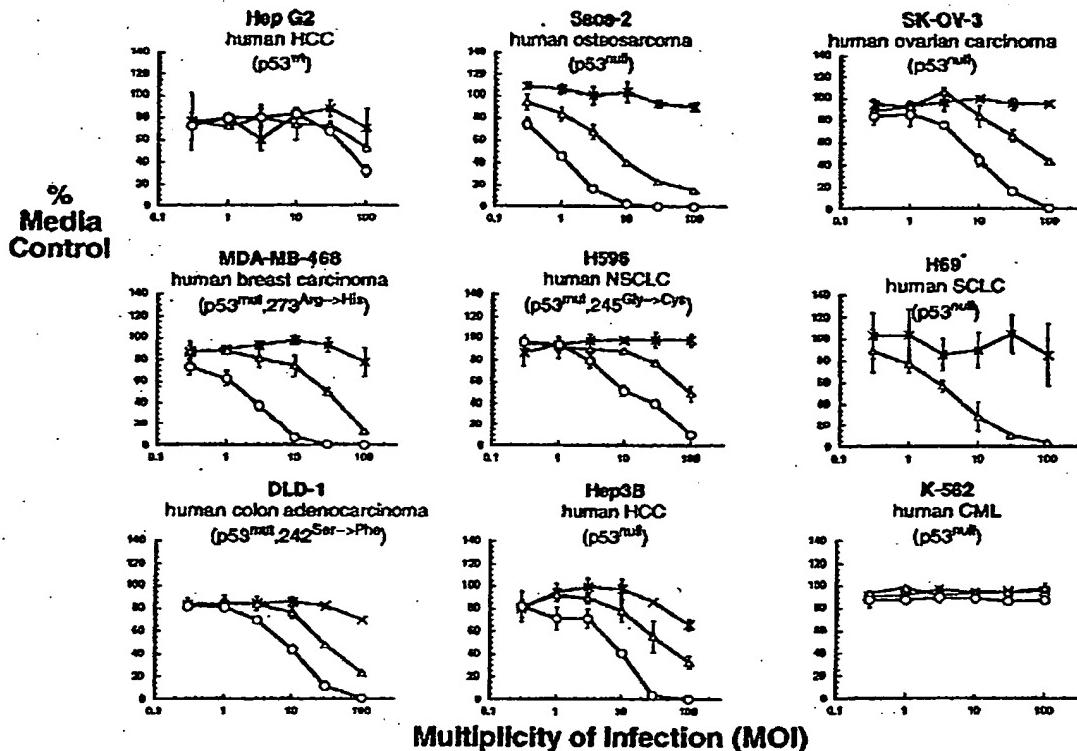
FIG. 4. p53-dependent inhibition of DNA synthesis in human tumor cell lines by A/M/N/53 and A/C/N/53. Nine different tumor cell lines were infected with either control adenovirus A/M/ (xx), or the p53-expressing A/M/N/53 (Δ) or A/C/N/53 (O) virus at increasing moi as indicated. Tumor type and p53 status are noted for each cell line (wt, wild type; null, no protein expressed; mut, mutant protein expressed). DNA synthesis was measured 72 hr post-infection as described in Materials and Methods. Results are from triplicate measurements at each dose (mean ± SD), and are plotted as % of media control versus moi. (*) H69 cells were only tested with A/M and A/M/N/53 virus.

pressed mutant p53 or failed to express p53 protein. We also found that in this assay, the A/C/N/53 construct was consistently more potent than the A/M/N/53. In Saos-2 (osteosarcoma) and MDA-MB468 (breast cancer) cells, nearly 100% inhibition of DNA synthesis was achieved with the A/C/N/53 construct at a moi as low as 10. At doses where inhibition by the control adenovirus is only 10–30%, we observed a 50–100% reduction in DNA synthesis using either p53 recombinant adenovirus. In contrast, we observed no significant p53-specific effect with either construct as compared to control virus in HEP G2 cells (hepatocarcinoma cell line expressing endogenous wild-type p53; Bressac et al., 1990), nor in the K562 (p53 null; Feinstein et al., 1992) leukemic cell line.

Tumorigenicity in nude mice

In a more stringent test of function for our p53 recombinant adenoviruses, we infected tumor cells ex vivo and then injected

the cells into nude mice to assess the ability of the recombinants to suppress tumor growth in vivo. Saos-2 cells infected with A/M/N/53 or control A/M virus at a moi of 3 or 30 were injected into opposite flanks of nude mice. Tumor sizes were then measured twice a week over an 8-week period. At a moi of 30, we did not observe any tumor growth in the p53-treated flanks in any of the animals, while the control treated tumors continued to grow (Fig. 5). The progressive enlargement of the control virus-treated tumors was similar to that observed in the buffer-treated control animals. We also observed a clear difference in tumor growth between the control adenovirus and the p53 recombinant at a moi of 3, although tumors from 2 out of the 4 p53-treated mice did start to show some growth after approximately 6 weeks (data not shown). Thus, the A/M/N/53 recombinant adenovirus is able to mediate p53-specific tumor suppression in an in vivo environment. We have also observed very similar results when infecting and injecting the NSCLC cell line H596, which expresses mutant p53 protein with the same viruses (unpublished observations).

Days post tumor cell implantation
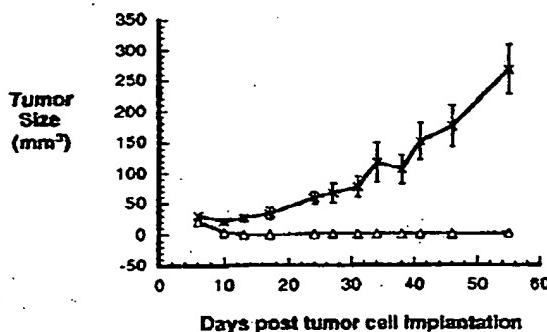
FIG. 5. Tumorigenicity of p53-infected Saos-2 cells in nude mice. Saos-2 cells were infected with either the control A/M virus or the p53 recombinant A/M/N/53 at moi = 30. Treated cells were injected subcutaneously into the flanks of nude mice, and tumor dimensions were measured (as described in Materials and Methods) twice per week for 8 weeks. Results are plotted as tumor size versus days post tumor cell implantation for both control A/M-(x) and A/M/N/53-(Δ) treated cells. Error bars represent the mean tumor size ± SEM for each group of 4 animals at each time point.

In vivo *expression of rAd/p53*

Although *ex vivo* treatment of cancer cells and subsequent injection into animals provided a critical test of tumor suppression, a more clinically relevant experiment is to determine if injected p53 recombinant adenovirus could infect and express p53 in established tumors *in vivo*. To address this, H69 (SCLC, p53^{null}) cells were injected subcutaneously into nude mice, and tumors were allowed to develop for 32 days. At this time, a single injection of 2 × 10^9 pfu of either A/C/53 or A/C/β-Gal adenovirus was injected into the peritumoral space surrounding the tumor. Tumors were then excised at either day 2 or day 7 following the adenovirus injection, and poly(A) RNA was isolated from each tumor. RT-PCR, using recombinant-p53 specific primers, was then used to detect p53 mRNA in the p53-treated tumors (Fig. 6, lanes 1, 2, 4, 5). No p53 signal was evident from the tumors excised from the β-Gal-treated animals (Fig. 6, lanes 3 and 6). Amplification with actin primers served as a control for the RT-PCR reaction (Fig. 6, lanes 7-9), while a plasmid containing the recombinant-p53 sequence served as a positive control for the recombinant-p53-specific band (Fig. 6, lane 10). This experiment demonstrates that a p53 recombinant adenovirus can specifically direct expression of p53 mRNA within established tumors following a single injection into the peritumoral space. It also provides evidence for *in vivo* viral persistence for at least 1 week following infection with a p53 recombinant adenovirus.

In vivo *efficacy*

To address the feasibility of gene therapy of established tumors, a tumor-bearing nude mouse model was used. H69 cells were injected into the subcutaneous space on the right flank of mice, and tumors were allowed to grow for 2 weeks. Mice then received peritumoral injections of buffer or recombinant virus
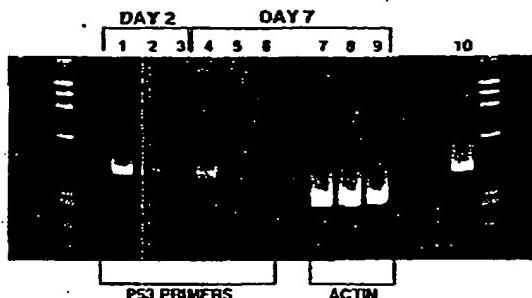
FIG. 6. Expression of rAd/p53 RNA in established tumors. H69 (SCLC) cells were injected subcutaneously into nude mice and allowed to develop tumors for 32 days until reaching a size of approximately 25-50 mm^3. Mice were randomized and injected peritumorally with 2 × 10^9 pfu of either control A/C/β-Gal or A/C/53 virus. Tumors were excised 2 and 7 days post injection, and poly(A) RNA was prepared from each tumor sample. RT-PCR was carried out using equal RNA concentrations and primers specific for recombinant p53 message. PCR amplification was for 30 cycles at 94°C 1 min, 55°C 1.5 min, 72°C 2 min, and a 10-min, 72°C final extension period in an Omnigen thermalcycler (Hybaid). The PCR primers used were a 5' Tripartite Leader cDNA (5'-CGCCACCGAGGGACCT-GAGCGAGTC-3') and a 3' p53 primer (5'-TTCTGGGAAGG-GACAGAAGA-3'). Lanes 1, 2, 4, and 5, p53-treated samples excised at days 2 or 7 as indicated; lanes 3 and 6, from β-Gal-treated tumors; lanes 7, 8, and 9, replicates of lanes 4, 5, and 6, respectively, amplified with actin primers to verify equal loading; lane 10, a positive control using a tripartite/p53 containing plasmid.

twice weekly for a total of 8 doses. In the mice treated with buffer or control A/M virus, tumors continued to grow rapidly throughout the treatment, whereas those treated with the A/M/N/53 virus grew at a greatly reduced rate (Fig. 7A). Although control animals treated with buffer alone had accelerated tumor growth as compared to either virus-treated group, we found no significant differences in body weight among the three groups during the treatment period (data not shown). Tumor ulceration in some animals limited the relevance of tumor size measurements after day 42. However, continued monitoring of the animals to determine survival time demonstrated a survival advantages for the p53-treated animals (Fig. 7B). The last of the control adenovirus-treated animals died on day 83, while buffer alone treated controls had all expired by day 56. In contrast, all 5 animals treated with the A/M/N/53 survived up to day 137 before the first animal in this group died (Fig. 7B). Two animals continue to survive at day 174. Together, our data indicate a p53-specific effect on both tumor growth and survival time in animals with established p53-deficient tumors.

DISCUSSION

Adenovirus vectors expressing p53

We have constructed recombinant human adenovirus vectors that are capable of expressing high levels of wild-type p53
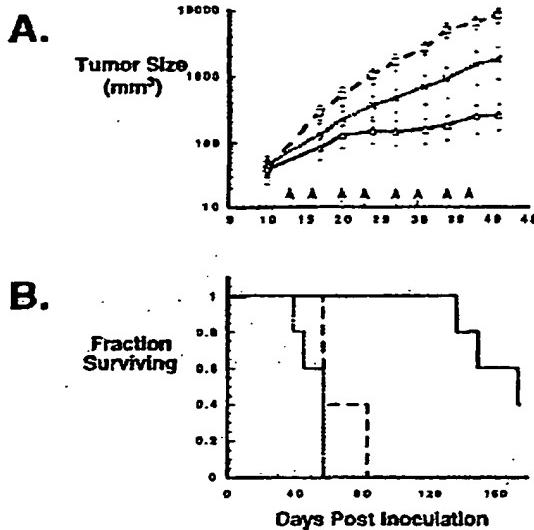
FIG. 7. *In vivo* tumor suppression and increased survival time with A/M/N/53. H69 (SCLC) tumor cells were injected subcutaneously into nude mice and allowed to develop for 2 weeks. Peritumoral injections of either buffer alone (☐), control A/M adenovirus (x), or A/M/N/53 (Δ) (both virus 2×10^9 pfu/injection) were administered twice per week for a total of 8 doses. Tumor dimensions were measured twice per week and tumor volume was estimated as described in Materials and Methods. A. Tumor size is plotted for each virus *versus* time (days) post inoculation of H69 cells. Error bars indicate the mean tumor size ± SEM for each group of 5 animals. Arrows indicate days of virus injections. B. Mice were monitored for survival and the fraction of mice surviving per group *versus* time post inoculation of buffer alone (---), control A/M (-- --), or A/M/N/53 (——) virus-treated H69 cells is plotted.

protein in a dose-dependent manner. Each vector contains deletions in the E1a and E1b regions that render the virus replication deficient (Challberg and Kelly, 1979; Horowitz, 1991). Of further significance is that these deletions include those sequences encoding the E1b 19- and 55-kD proteins. The 19-kD protein is reported to be involved in inhibiting apoptosis (Rao *et al.*, 1992; White *et al.*, 1992), whereas the 55-kD protein is able to bind wild-type p53 protein (Sarnow *et al.*, 1982; Heuvel *et al.*, 1990). By deleting these adenoviral sequences, we remove potential inhibitors of p53 function through direct binding to p53 or potential inhibition of p53-mediated apoptosis. We have created additional constructs that have had the remaining 3' E1b sequence, including all protein IX coding sequence, deleted as well. Although this has been reported to reduce the packaging size capacity of adenovirus to approximately 3 kb, less than wild-type virus (Ghosh-Choudhury *et al.*, 1987), these constructs are also deleted in the E3 region so that the A/M/N/53 and A/C/N/53 constructs are well within this size range. By deleting the pIX region, adenoviral sequences homologous to those contained in 293 cells are reduced to approximately 300 bp, decreasing the chances of regenerating replication-compe-

tent, wild-type adenovirus through recombination. Constructs lacking pIX coding sequence appear to have equal efficacy and drive equivalent levels of p53 protein expression as those with pIX (unpublished observations).

p53/Adenovirus efficacy in vitro

In concordance with a strong dose dependency for expression of p53 protein in infected cells, we have also demonstrated a dose-dependent, p53-specific inhibition of tumor cell growth by our recombinants. We were able to inhibit cell division, demonstrated by the inhibition of DNA synthesis, in a wide variety of tumor cell types known to lack wild-type p53 protein expression. Bacchetti and Graham (1993) recently reported p53-specific inhibition of DNA synthesis in the ovarian carcinoma cell line SKOV-3 by a p53 recombinant adenovirus in similar experiments. In addition to ovarian carcinoma, we have demonstrated that additional human tumor cell lines, representative of clinically important human cancers and including lines overexpressing mutant p53 protein, can also be growth inhibited by our p53 recombinants. At moi values where the A/C/N/53 recombinant is 90–100% effective in inhibiting DNA synthesis in these tumor types, control adenovirus-mediated suppression is less than 20%.

Although Feinstein *et al.* (1992) reported that reintroduction of wild-type p53 could induce differentiation and increase the proportion of cells in G_1 versus $S + G_2$ for leukemic K562 cells, we found no p53-specific effect in this line. Horvath and Weber (1988) have reported that human peripheral blood lymphocytes are highly nonpermissive to adenovirus infection. In separate experiments, we found that we were not able to infect the nonresponding K562 cells significantly with recombinant A/C/β-Gal adenovirus, while other cell lines, including the control Hep G2 line and those showing a strong p53 effect, were readily infectable (Harris *et al.*, in preparation). Thus, at least part of the variability of efficacy would appear to be due to variability of infection, although other factors may be involved as well. For example, Chen *et al.* (1991) reported that wild-type p53 can suppress tumorigenicity without inhibiting the growth rate of some tumor lines. Alternatively, mutations of regulatory proteins acting downstream from p53 may also exist in some tumor cell lines, limiting the effect of p53 treatment. The lack of a p53-specific effect in the wild-type control cell line Hep G2 is encouraging, suggesting that overexpression of wild-type p53 over endogenous background levels may have only minor effects in normal cells infected with the recombinant.

The ability to treat human cancer cells *ex vivo* and suppress their growth *in vivo* when implanted into an animal is an important step toward identifying promising gene therapy candidates. The results observed with the A/M/N/53 virus in Fig. 5 demonstrates that complete suppression is possible in an *in vivo* environment. The resumption of tumor growth in 2 out of the 4 p53-treated animals at the lower moi most likely resulted from a small percentage of cells not initially infected with the p53 recombinant at this dose. We did not analyze the resulting tumors for the presence of adenoviral genomes. The complete suppression seen with A/M/N/53 at the higher dose, however, shows that the ability of tumor growth to recover can be overcome.

p53/Adenovirus in vivo efficacy

Work presented here and by other groups (Chen et al., 1990; Takahashi et al., 1992) have shown that human tumor cells lacking expression of wild-type p53 can be treated ex vivo with p53 and result in suppression of tumor growth when the treated cells are transferred into an animal model. This report presents the first evidence of tumor suppressor gene therapy of an in vivo established tumor, resulting in both suppression of tumor growth and increased survival time. Delivery to tumor cells did not rely on direct injection into the tumor mass. Rather, p53 recombinant adenovirus was injected into the peritumoral space, and p53 mRNA expression was detected within the tumor. p53 expressed by the recombinants was functional and strongly suppressed tumor growth as compared to that of control, non-p53-expressing adenovirus-treated tumors. However, both p53 and control virus-treated tumor groups showed tumor suppression as compared to buffer-treated controls. It has been demonstrated that local expression of tumor necrosis factor (TNF), interferon-γ (IFN-γ), interleukin (IL)-2, IL-4, or IL-7 can lead to T-cell-independent transient tumor suppression in nude mice (Hoch et al., 1992). Exposure of monocytes to adenovirus results in the release of TNF, and adenovirus virions are also weak inducers of IFN-α/β (for review, see Gooding and Wold, 1990). Therefore, it is not surprising that we observed some tumor suppression in nude mice even with the control adenovirus. We did not observe this virus-mediated tumor suppression in the ex vivo control virus-treated Saos-2 tumor cells described earlier. The p53-specific in vivo tumor suppression was dramatically demonstrated by continued monitoring of the animals in Fig. 7. The survival time of the p53-treated mice was significantly increased, with 5 out of 5 animals still alive more than 135 days after tumor cell inoculation compared to 0 out of 5 adenovirus control-treated animals. Two out of 5 mice continue to survive beyond day 170, more than twice the survival time of the longest-lived control virus and buffer-treated animals. The surviving animals still exhibit growing tumors, which may reflect cells not initially infected with the p53 recombinant adenovirus. Higher or more frequent dosing schedules may address this. In addition, promoter shutoff (Palmer et al., 1991) or additional mutations may have rendered these cells resistant to the p53 recombinant adenovirus treatment.

Implications for gene therapy

There will be over one million new cases of cancer diagnosed this year, and half that number of cancer-related deaths (American Cancer Society, 1993). p53 mutations are the most common genetic alteration associated with human cancers, occurring in 50–60% of human cancers (Bartek et al., 1991; Hollstein et al., 1991; Levine, 1993). The goal of gene therapy in treating p53-deficient tumors is to reinstate a normal, functional copy of the wild-type p53 gene so that control of cellular proliferation is restored. p53 plays a central role in cell cycle progression, arresting growth so that repair or apoptosis can occur in response to DNA damage. The possibility of using p53/adenovirus to drive tumor cells into the apoptotic pathway is intriguing. Wild-type p53 has recently been identified as a necessary component for apoptosis induced by irradiation or

treatment with some chemotherapeutic agents (Lowe et al., 1993a,b). Due to the high prevalence of p53 mutations in human tumors, it is possible that tumors which have become refractory to chemotherapy and irradiation treatments may have become so due in part to the lack of wild-type p53. By resupplying functional p53 to these tumors, it is possible that they will now become susceptible to apoptosis normally associated with the DNA damage induced by radiation and chemotherapy.

One of the critical points in successful human tumor suppressor gene therapy is the ability to affect a significant fraction of the cancer cells. Toward that goal, recombinant adenoviruses have distinct advantages over other gene delivery methods (for review, see Siegfried, 1993). Adenoviruses have never been shown to induce tumors in humans and have been safely used as live vaccines (Straus, 1984). Replication-deficient recombinant adenoviruses can be produced by replacing the E1 region necessary for replication with the target gene. Adenovirus does not integrate into the human genome as a normal consequence of infection, thereby greatly reducing the risk of insertional mutagenesis possible with retrovirus or AAV vectors. This lack of stable integration also leads to an additional safety feature in that the transferred gene effect will be transient, as the extrachromosomal DNA will be gradually lost with continued division of normal cells. Stable, high-titer recombinant adenovirus can be produced at levels not achievable with retrovirus or AAV, allowing enough material to be produced to treat a large patient population. Others have shown that adenovirus-mediated gene delivery has a strong potential for gene therapy for diseases such as cystic fibrosis (Rosenfeld et al., 1992; Rich et al., 1993) and α₁-antitrypsin deficiency (Lemarchand et al., 1992). Although other alternatives for gene delivery, such as cationic liposome-DNA complexes, are also currently being explored, none as yet appear as effective as adenovirus-mediated gene delivery.

Here, we have shown that recombinant adenoviruses expressing wild-type p53 can efficiently inhibit DNA synthesis and suppress the growth of a broad range of human tumor cell types, including clinically relevant targets. Furthermore, we have shown that the recombinant adenoviruses can express p53 in an in vivo established tumor without relying on direct injection into the tumor or prior ex vivo treatment of the cancer cells. The p53 expressed is functional and effectively suppressed tumor growth in vivo and significantly increased survival time in a nude mouse model of human lung cancer. Although further studies are needed to ensure the safety of this method of gene delivery and address possible problems of immune responses, the data presented here strongly support the concept of adenovirus-mediated p53 gene therapy of p53-deficient tumors in humans.

ACKNOWLEDGMENTS

p53 RECOMBINANT ADENOVIRUS GENE THERAPY OF CANCER

1087

REFERENCES

AMERICAN CANCER SOCIETY. (1993). Cancer Facts and Figures.

BACCHETTI, S., and GRAHAM, F. (1993). Inhibition of cell proliferation by an adenovirus vector expressing the human wild type p53 protein. Int. J. Oncol. 3, 781–788.

BAKER, S.J., MARKOWITZ, S., FEARON, E.R., WILLSON, J.K.V., and VOGELSTEIN, B. (1990). Suppression of human colorectal carcinoma cell growth by wild-type p53. Science 249, 912–915.

BARTEK, J., BARTKOVA, J., VOJTESEK, B., STASKOVA, Z., LUKAS, J., REJTHAR, A., KOVARIK, J., MIDGLEY, C.A., GANNON, J.V., and LANE, D.P. (1991). Aberrant expression of the p53 oncoprotein is a common feature of a wide spectrum of human malignancies. Oncogene 6, 1699–1703.

BRESSAC, B., GALVIN, K.M., LIANG, T.J., ISSELBACHER, K.J., WANDS, J.R., and OZTURK, M. (1990). Abnormal structure and expression of p53 gene in human hepatocellular carcinoma. Proc. Natl. Acad. Sci. USA 87, 1973–1977.

CHALLBERG, M.D., and KELLY, T.J. (1979). Adenovirus DNA replication in vitro. Biochemistry 76, 655–659.

CHEN P.L., CHEN Y., BOOKSTEIN R., and LEE W.H. (1990). Genetic mechanisms of tumor suppression by the human p53 gene. Science 250, 1576–1580.

CHEN, Y., CHEN, P.L., ARNAIZ, N., GOODRICH, D., and LEE, W.H. (1991). Expression of wild-type p53 in human A673 cells suppresses tumorigenicity but not growth rate. Oncogene 6, 1799–1805.

CHENG, J., YEE, J.K., YEARGIN, J., FRIEDMANN, T., and HAAS, M. (1992). Suppression of acute lymphoblastic leukemia by the human wild-type p53 gene. Cancer Res. 52, 222–226.

DILLER, L., KASSEL, J., NELSON, C.E., GRYKA, M.A., LITWAK, G., GEBHARDT, M., BRESSAC, B., OZTURK, M., BAKER, S.J., VOGELSTEIN, B., and FRIEND, S.H. (1990). p53 functions as a cell cycle control protein in osteosarcomas. Mol. Cell. Biol. 10, 5772–5781.

FEINSTEIN, E., GALE, R.P., REED, J., and CANAANI, E. (1992). Expression of the normal p53 gene induces differentiation of K562 cells. Oncogene 7, 1853–1857.

GHOSH-CHOUDHURY, G., HAJ-AHMAD, Y., and GRAHAM, F.L. (1987). Protein IX, a minor component of the human adenovirus capsid, is essential for the packaging of full length genomes. EMBO J. 6, 1733–1739.

GOODING, L.R., and WOLD, W.S.M. (1990). Molecular mechanisms by which adenoviruses counteract antiviral immune defenses. Crit. Rev. Immunol. 10, 53–71.

GRAHAM, F.L., and VAN DER EBB, A.J. (1973). A new technique for the assay of infectivity of human adenovirus 5 DNA. Virology 52, 456–467.

GRAHAM, F.L., SMILEY, J., RUSSELL, W.C., and NAIRN, R. (1977). Characteristics of a human cell line transformed by DNA from human adenovirus type 5. J. Gen. Virol. 36, 59–74.

GRAHAM, F.L., and PREVEC, L. (1991). Manipulation of adenovirus vectors. In Methods in Molecular Biology, vol. 7. Gene Transfer and Expression Protocols. E.J. Murray, ed. (The Humana Press Inc., Clifton NJ) pp. 109–128.

HEUVEL, S.J.L., LAAR, T., KAST, W.M., MELIEF, C.J.M., ZANTEMA, A., and VAN DER EB, A.J. (1990). Association between the cellular p53 and the adenovirus 5 E1B-55kd proteins reduces the oncogenicity of Ad-transformed cells. EMBO J. 9, 2621–2629.

HOCK, H., DORSCH, M., KUZENDORP, U., QIN, Z., DIAMANTSTEIN, T., and BLANKENSTEIN, T. (1992). Mechanisms of rejection induced by tumor cell-targeted gene transfer of interleukin 2, interleukin 4, interleukin 7, tumor necrosis factor, or interferon γ. Proc. Natl. Acad. Sci. USA 90, 2774–2778.

HOLLSTEIN, M., SIDRANSKY, D., VOGELSTEIN, B., and HARRIS C. (1991). p53 mutations in human cancers. Science 253, 49–53.

HOROWITZ, M.S. (1991). Adenoviridae and their replication. In Fields Virology. B.N. Fields, ed. (Raven Press, New York) pp. 1679–1721.

HORVATH, J., and WEBER, J.M. (1988). Nonpermissivity of human peripheral blood lymphocytes to adenovirus type 2 infection. J. Virol. 62, 341–345.

HSU, I.C., TOKIWA, T., BENNETT, W., METCALF, R.A., WELSH, J.A., SUN, T., and HARRIS, C.C. (1993). p53 gene mutation and integrated hepatitus B viral DNA sequences in human liver cancer cell lines. Carcinogenesis 14, 987–992.

JONES, N., and SHENK, T. (1979). Isolation of adenovirus type 5 host range deletion mutants defective for transformation of rat embryo cells. Cell 17, 683–689.

KEURBITZ, S.J., PLUNKETT, B.S., WALSH, W.V., and KASTAN, M.B. (1992). Wild-type p53 is a cell cycle checkpoint determinant following irradiation. Proc. Natl. Acad. Sci. USA 89, 7491–7495.

LANE, D.P. (1992). p53, guardian of the genome. Nature 358, 15–16.

LEMARCHAND, P., JAFFE, H.A., DANEL, C., CID, M.C., KLEINMAN, H.K., STRATFORD-PERRICAUDET, L.D., PERRICAUDET, M., PAVIRANI, A., LECOCQ, J.P., and CRYSTAL, R.G. (1992). Adenovirus-mediated transfer of a recombinant human α₁-antitrypsin cDNA to human endothelial cells. Proc. Natl. Acad. Sci. USA 89, 6482–6486.

LEVINE, A.J. (1993). The tumor suppressor genes. Annu. Rev. Biochem. 62, 623–651.

LOWE, S.W., RULEY, H.E., JACKS, T., and HOUSMAN, D.E. (1993a). p53-dependent apoptosis modulates the cytotoxicity of anticancer agents. Cell 74, 957–967.

LOWE, S.W., SCHMITT, E.M., SMITH, S.W., OSBORNE, B.A., and JACKS, T. (1993b). p53 is required for radiation-induced apoptosis in mouse thymocytes. Nature 362, 847–852.

MERCER, W.E., SHIELDS, M.T., AMIN, M., SUAVE, G.J., APPELLA, E., ROMANO, J.W., and ULLRICH, S.J. (1990). Negative growth regulation in a glioblastoma tumor cell line that conditionally expresses human wild-type p53. Proc. Natl. Acad. Sci. USA 87, 6166–6170.

PALMER, T.D., ROSMAN, G.J., OSBORNE, W.R., and MILLER, A.D. (1991). Genetically modified skin fibroblasts persist long after transplantation but gradually inactivate introduced genes. Proc. Natl. Acad. Sci. USA 88, 1330–1334.

RAO, L., DEBBAS, M., SABBATINI, P., HOCKENBERY, D., KORSMEYER, S., and WHITE, E. (1992). The adenovirus E1A proteins induce apoptosis, which is inhibited by the E1B 19-kDa and Bcl-2 proteins. Proc. Natl. Acad. Sci. USA 89, 7742–7746.

RICH, D.P., COUTURE, L.A., CARDOZA, L.M., GUIGGIO, V.M., ARMENTANO, D., ESPINO, P.C., HEHIR, K., WELSH, M.J., SMITH, A.E., and GREGORY, R.J. (1993). Development and analysis of recombinant adenoviruses for gene therapy of cystic fibrosis. Hum. Gene Ther. 4, 461–476.

ROSENFELD, M.A., YOSHIMURA, K., TRAPNELL, B.C., YONEYAMA, K., ROSENTHAL, E.R., DALEMANS, W., FUKAYAMA, M., BARGON, J., STIER, L.E., STRATFORD-PERRICAUDET, L., PERRICAUDET, M., GUGGINO, W.B., PAVIRANI, A., LECOCQ, J.P., and CRYSTAL, R.G. (1992). In vivo transfer of the human cystic fibrosis transmembrane conductance regulator gene to the airway epithelium. Cell 68, 143–155.

SAMBROOK, J., FRITSCH, E.F., and MANIATIS, T. (1989). Molecular Cloning: A Laboratory Manual. (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY).

SARNOW, P., HO, Y.S., WILLIAMS, J., and LEVINE, A.J. (1982).

Adenovirus E1b-58 kd tumor antigen and SV40 large tumor antigen are physically associated with the same 54 kd cellular protein in transformed cells. Cell 28, 387–394.

SHAW, P., BOVEY, R., TARDY, S., SAHLI, R., SORDAT, B., and COSTA, J. (1992). Induction of apoptosis by wild-type p53 in a human colon tumor-derived cell line. Proc. Natl. Acad. Sci. USA 89, 4495–4499.

SIEGFRIED, W. (1993). Perspectives in gene therapy with recombinant adenoviruses. Exp. Clin. Endocrinol. 101, 7–11.

STRAUS, S.E. (1984). Adenovirus infections in humans. In The Adenoviruses. H.S. Ginsberg, ed. (Plenum Press, New York) pp. 451–496.

TAKAHASHI, T., NAU, M.M., CHIBA, I., BIRRER, M.J., ROSENBERG, R.K., VINOCOUR, M., LEVITT, M., PASS, H., GAZDAR, A.F., and MINNA, J.D. (1989). p53: A frequent target for genetic abnormalities in lung cancer. Science 246, 491–494.

TAKAHASHI, T., CARBONE, D., TAKAHASHI, T., NAU, M.M., HIDA, T., LINNOILA, I., UEDA, R., and MINNA, J.D. (1992). Wild-type but not mutant p53 suppresses the growth of human lung cancer cells bearing multiple genetic lesions. Cancer Res. 52, 2340–2343.

THIMMAPPAYA, B., WEINBERGER, C., SCHNEIDER, R.J., and SHENK, T. (1982). Adenovirus VAI RNA is required for efficient translation of viral mRNAs at late times aftr infection. Cell 31, 543–551.

WANG, A.M., DOYLE, M.V., and MARK, D.F. (1989). Quantitation of mRNA by the polymerase chain reaction. Proc. Natl. Acad. Sci. USA 86, 9717–9721.

WHITE, E., SABBATINI, P., DEBBAS, M., WOLD, W.S.M., KUSHER, D.L., and GOODING, L.R. (1992). The 19-kilodalton adenovirus E1B transforming protein inhibits programmed cell death and prevents cytolysis by tumor necrosis factor. Mol. Cell. Biol. 12, 2570–2580.

YONISH-ROUACH, E., RESNITZKY, D., LOTEM, J., SACHS, L., KIMCHI, A., and OREN, M. (1991). Wild-type p53 induces apoptosis of myeloid leukaemic cells that is inhibited by interleukin-6. Nature 352, 345–347.

Address reprint requests to:
Dr. Ken N. Wills
CANJI, Inc.
3030 Science Park Road
Suite 302
San Diego, CA 92121